2008

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Keywords
Compensatory growth; cotyledon plate meristem; *Cucurbita pepo* (zucchini); cytokinins; hormonal interactions; senescence.

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ABSTRACT

The jasmonates are well studied in the context of plant defence but increasingly are also recognised as playing roles in development. In many systems, jasmonates antagonise the effects of cytokinins. The aim of the present work was to elucidate interactions between methyl jasmonate and cytokinin (benzyladenine) in regulating growth of zucchini (*Cucurbita pepo* L., cv. Cocozelle, var. Tripolis) cotyledons, taking advantage of the ability to simultaneously quantify cell enlargement and division from paradermal sections of the first palisade layer. Growth regulators were applied to cotyledons, excised from dry seeds and grown in darkness. Cytokinin stimulated expansion and division whereas, surprisingly, jasmonate stimulated expansion but inhibited division. Jasmonate antagonised the stimulating effect of cytokinin on division but worked cooperatively with cytokinin in increasing expansion. However, expansion with jasmonate was more isotropic than with cytokinin. Jasmonate also stimulated the loss of cellular inclusions and soluble protein. Soluble proteins revealed a partial antagonism between jasmonate and cytokinin. These results illustrate the complex interplay between jasmonates and cytokinin in the regulatory network of cotyledon development following germination.

INTRODUCTION

Following germination, cotyledons supply the seedling with nutrients that were stored during embryonic development. Cotyledons of the epigeal type, once exposed to light, expand and photosynthesise, providing an additional source of fixed carbon for seedling establishment. Because cotyledon expansion initially requires stored reserves that might otherwise be used for other seedling organs, it is possible that this expansion is subject to a complicated net of hormonal interactions, tuning growth to the environment. While cytokinin has long been known as the major, hormonal regulator of cotyledon development (reviewed in Mok & Mok 2001), there is evidence that jasmonic acid and its methyl ester may also be involved, possibly as antagonists to cytokinin (Ueda & Kato 1982; Ananieva & Ananiev 1999; Mukherjee *et al.* 2002). Jasmonates are primarily known for their roles in response to herbivory, which have been well studied; however, it is clear that they also play a role in development (Wasternack & Hause 2002). In terms of growth and division, exogenously applied jasmonate can be either inhibitory (Ueda & Kato 1982; Swiatek *et al.* 2002, 2003) or stimulatory (Kondo *et al.* 2002; Cenzano *et al.* 2003; Capitani *et al.* 2005). Jasmonates are present in relative abundance at the shoot apex (Sembdner & Klose 1985), young leaves and developing fruits (Fan *et al.* 1997; Kondo *et al.* 2002), which led to the proposal that they participate in development of the meristem. However, effects of exogenous jasmonate on cell division parameters during shoot meristem development could not always be detected (*e.g.* Cenzano *et al.* 2003). The nature of the control that jasmonates exert on growth and morphogenesis remains to be elucidated.

The etiolated epigeal cotyledon is a useful system for studying the control of expansion and division because vigorous growth occurs in the excised condition and in the dark, removing complications from interactions with the rest of the seedling, photosynthesis or photomorphogenesis. Etiolated cotyledons are also useful because light

is known to interfere with some effects of cytokinins. Cotyledon cells of many species divide frequently following germination, which gives rise to the name 'plate meristem' for the dividing tissues of the cotyledon, and implies that the cotyledon can be compared to the more complex shoot and root apical meristems. For quantifying both expansion and division in epigeal cotyledons, Stoynova-Bakalova (2007) proposed the use of the first layer of palisade tissue from zucchini, because of the relatively homogenous histology and high division activity, which occurs exclusively in the anticlinal plane.

Here, we have used the first palisade layer of zucchini cotyledons, isolated from dry seeds and cultured in darkness, to image and quantify the effects of exogenous methyl jasmonate on cell division and expansion and to characterise interactions between jasmonate and cytokinin in the control of cotyledon development.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *Cucurbita pepo* L., cv. Cocozelle var. Tripolis (zucchini) were stripped of their seed coats, and cotyledons isolated from the embryonic axes of the seed were placed in covered Petri dishes on filter paper soaked with distilled water, supplemented with either methyl jasmonate, benzyladenine (N^6-benzylaminopurine), or both. Benzyladenine was obtained from Sigma Chemical Co. (St Louis, MO, USA) and methyl jasmonate was obtained from Serva (Heidelberg, Germany). Cotyledons were cultivated in the dark at 28 °C. Time-course experiments were made with seeds from a different seed batch than those used for all other experiments.

Growth analysis, light microscopy and image processing

To determine fresh and dry weights, on the sixth day of treatment, 12 cotyledons per treatment were weighed separately and oven dried at 105 °C until reaching a constant weight (between 20 to 24 h).

Cotyledon growth was characterised by an increase in area of at least 20 cotyledons in each of three independent experiments. To measure area, cotyledons were carefully flattened and scanned (Epson Perfection 2480 Photoscanner, Matsumoto-shi City, Japan). The cotyledon images were taken with a digital camera.

For microscopy, samples were taken from the central area of the cotyledon lamina, within 3 mm of the centre of the cotyledon. The cotyledon pieces were fixed in 3% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2, and embedded in low viscosity Spurr's epoxy-resin. Semi-thin sections, cut on an ultramicrotome (Tesla BS 490, Brno, Czech Republic), were stained with 0.01% (w/v) toluidine blue and observed under a light microscope (Carl Zeiss, Jena, Germany). Paradermal sections through the first palisade layer, as well as transverse sections through all cotyledon cell layers, were collected. Microscopic images of the palisade layer were captured and saved on a digital image processor (International Micro-Vision Inc., Redwood City, CA, USA).

The number of completed cell divisions in the first palisade layer during cotyledon growth was determined in two related ways: first, by increase in the mean number of daughter cells present in clusters ('mother cells'); and second, by increase in the mean number of palisade cells per unit area of the enlarging cotyledon. Cell number (of about 600 cells from the first palisade layer) was evaluated in paradermal sections, obtained from five cotyledons per treatment. Cell number and cell and cotyledon areas were measured using the 3DDoctor software (Able Software Corp., Lexington, MA, USA).

Protein extraction, quantification and SDS-PAGE

Changes in soluble protein were evaluated on the third day of cotyledon development, when palisade cells are active in division and enlargement. Cotyledon palisade tissue (*c.* 100 mg), dissected from cotyledons by hand with a razor, was homogenised with quartz sand in 300 µl of cold homogenisation buffer (60 mM Tris-HCl, pH 6.8; containing 5 mM EDTA and 14 mM 2-mercaptoethanol), in a pre-cooled mortar and pestle. The homogenates were centrifuged at 13,000 *g* for 20 min. The supernatant was used as the soluble protein extract. Concentrations were measured using a dye binding assay (Bradford 1976) with bovine serum albumin grade V (Sigma) as a standard. Protein content was expressed per g fresh weight. To identify changes in protein content and polypeptide pattern, equal volumes of the samples, extracted from equal fresh weights of tissues, were loaded. SDS-PAGE was performed as described by Okadjima *et al.* (1993) using 5% and 12% polyacrylamide in the stacking and resolving gels, respectively. Proteins were detected by Coomassie Blue staining. Proteins were extracted in replicate experiments and the consistency of the profiles between experiments was verified by analysing scanned images of the gels with Gel-Pro-Analyzer 3.1 (Media Cybernetics Inc., Bethesda, MD USA).

RESULTS

All experiments reported here used cotyledons excised from dry seeds and cultured in darkness. Under these conditions, cotyledon expansion was stimulated by methyl jasmonate in a concentration-dependent manner (Fig. 1). The extent of the stimulation varied among seed batches but was always significant. At the highest concentrations, a few brown spots were observed on a limited number of cotyledons. Cotyledon expansion was also stimulated by benzyladenine, particularly when combined with the higher concentrations of methyl jasmonate (*i.e.* 100 and 1000 µM). The strength of the effect was essentially independent of seed batch.

Methyl jasmonate, despite stimulating cotyledon expansion, decreased cell division. By the fourth day of treat-

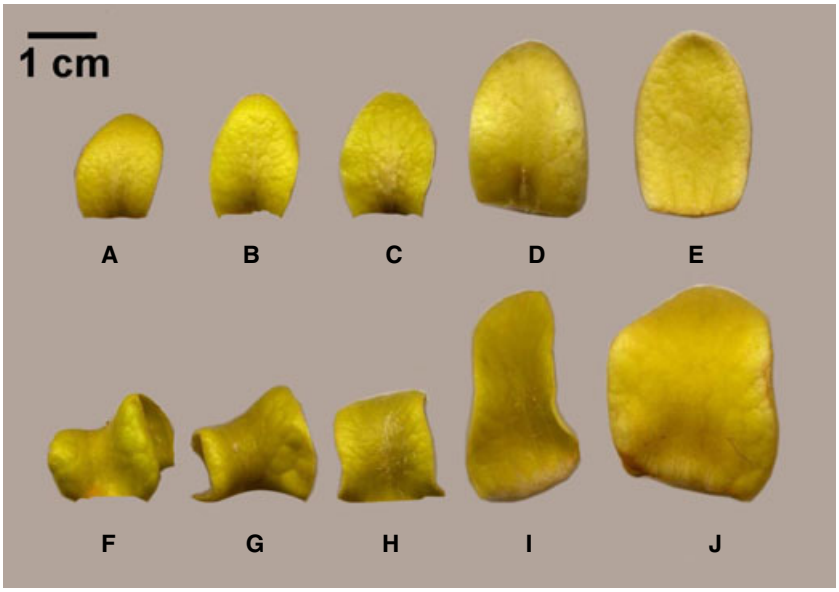

Fig. 1. Cultivated cotyledons, day 6 development. A: Control. B: 1 μM MeJA. C: 10 μM MeJA. D: 100 μM MeJA. E: 1000 μM MeJA. F: 10 μM BA. G: 10 μM BA + 1 μM MeJA. H: 10 μM BA + 10 μM MeJA. I: 10 μM BA + 100 μM MeJA. J: 10 μM BA + 1000 μM MeJA.

ment, many palisade cells in control cotyledons have divided once, thus forming two-celled clusters (arrow, Fig. 2A); however, on 100 μM methyl jasmonate, only a few clusters had formed (Fig. 2C). In contrast, 10 μM benzyladenine provoked almost all palisade cells to divide and, by day 4, several rounds of division had occurred so that clusters formed with many more than two cells (Fig. 2B). When methyl jasmonate and cytokinin were given together, the effect on division was intermediate, with many two-celled clusters but few clusters with more than two cells (Fig. 2D).

To assess the effects on cell division, we assayed the average number of cells per cluster on each day of treatment, up to 7 day (Fig. 3). In controls, divided cells first appeared on day 3 and cell number per cluster became constant by day 5. Methyl jasmonate at 10 μM chiefly appeared to slow the rate of increase in cells per cluster, whereas 100 μM allowed division on day 2 but suppressed it thereafter, and 1 mM blocked division completely. In contrast, benzyladenine increased the number of cells per cluster considerably, and advanced by one day the onset of division as well as its cessation. Interestingly, 10 μM methyl jasmonate prevented the earlier onset of division caused by the cytokinin but only slightly reduced the final number of cells per cluster. Higher levels of jasmonate given with the cytokinin progressively reduced the maximal number of cells per cluster.

Additional differences between the treatments are illustrated in paradermal sections of palisade tissue in cotyledons cultivated for 6 days (Fig. 4), when cell proliferation had ceased in all treatments, as seen in Fig. 3. In control cotyledons, cell division resulted in the formation of an average of four daughter cells per cluster (Fig. 4A). For cytokinin-treated cotyledons, because proliferation had stopped 2 days previously (Fig. 3) and daughter cells had begun to separate, the clusters of daughter cells were not as clear on day 6 (Fig. 4B) as on day 4 (Fig. 2B). In contrast, in cotyledons treated with 100 μM methyl jasmonate, two-cell clusters, although rare, remained distinct (Fig. 4C), suggesting that this hormone retarded separation of the daughter cells. At the higher concentration of jasmonate (1 mM), no divisions were detected (Fig. 4E).

Treatments with both compounds increased the heterogeneity of the population, both in terms of the number of cells per cluster and cell size (Fig. 4D and F). Heterogeneity was particularly pronounced in the epidermis. The enhanced enlargement of intercellular spaces on the sixth day was especially pronounced in cotyledons grown in the presence of 100 μM methyl jasmonate and 10 μM benzyladenine, possibly due to insufficient coordination of enlargement between sister cells (Fig. 4D). Cotyledons treated with methyl jasmonate alone or with the cytokinin differed from controls and cytokinin-treated samples in that the cells appeared optically almost empty, suggesting that the stored cellular reserves had been metabolised to a greater extent.

For comparison with division, we also quantified cotyledon area. Figure 5 plots the area when average cell number per cluster became constant (black portion of bar) as well as the final area (total bar height). Both methyl jasmonate and benzyladenine stimulated cotyledon expansion, but for the cytokinin most of the expansion occurred while cells were dividing, whereas for jasmonate most of the expansion followed division. Interestingly, although methyl jasmonate stimulated expansion in area, transverse sections show that the palisade layers were shorter after jasmonate treatment than in controls or cytokinin-treated cotyledons (Fig. 6). Similarly, stimulation of area expansion by methyl jasmonate did not alter the aspect ratio (length to width) of the cotyledon,

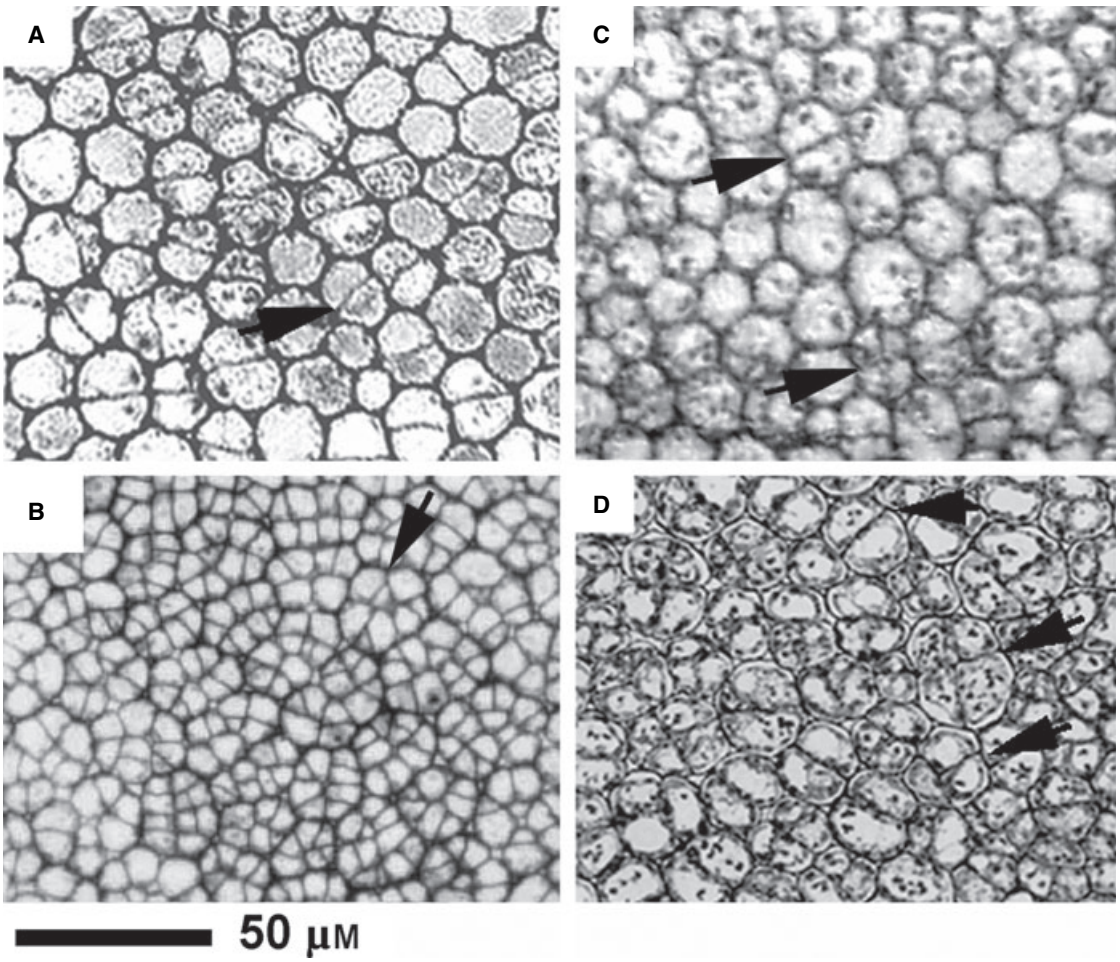

Fig. 2. Micrographs of paradermal sections of the first palisade layer on day 4 of treatment. A: Control. B: 10 μM benzyladenine. C: 100 μM MeJA. D: 100 μM MeJA and 10 μM BA. Arrows = clusters of daughter cells.

whereas with cytokinin growth in length was favoured (Fig. 7). Taking all three dimensions into account, methyl jasmonate induced expansion that was more isotropic in character than that of control or cytokinin-treated material.

To determine the overall effect of the tested compounds on total growth, cotyledon fresh and dry weights were measured (Table 1). Jasmonate promoted water uptake compared to the control but less strongly than did benzyladenine, which is consistent with the thinner lamina on jasmonate-treated coltyledons (Fig. 6). The combined treatment was clearly more effective than either alone. Interestingly, dry weight was completely maintained on the cytokinin whereas it decreased significantly on methyl jasmonate, which is consistent with the faster disappearance of cell contents observed in paradermal sections (Fig. 4). The combined treatment had an intermediate effect.

To more fully characterise effects of the two hormones on stored reserves, we assayed the protein content. Ten micromolar of benzyladenine reduced the amount of soluble protein by about 13% compared to the control, whereas methyl jasmonate at the same concentration induced a slight increase (by 7%) in protein content (Fig. 8). After treatment with jasmonate alone at higher concentrations (100 and 1000 μM), soluble proteins decreased compared to the control, by 28% and 35%, respectively. When applied together, benzyladenine prevented the decrease induced by jasmonate.

Soluble proteins were extracted from palisade tissue of cotyledons treated for 3 day, separated by denaturing polyacrylamide gel electrophoresis, and stained with Coomassie blue. Figure 9 shows a representative gel; the analyses of scanned gels from three different experiments found that most changes between treatments were consistent (not shown). In a concentration-dependent manner, methyl jasmonate induced the synthesis of several proteins (69, 60, and 43 kDa) that were not detected in the water- or cytokinin-treated cotyledons, and increased the abundance of two proteins (97.4 and 53 kDa) present in the other treatments. Additionally, jasmonate decreased the abundance of a band at 55 kDa, the same relative

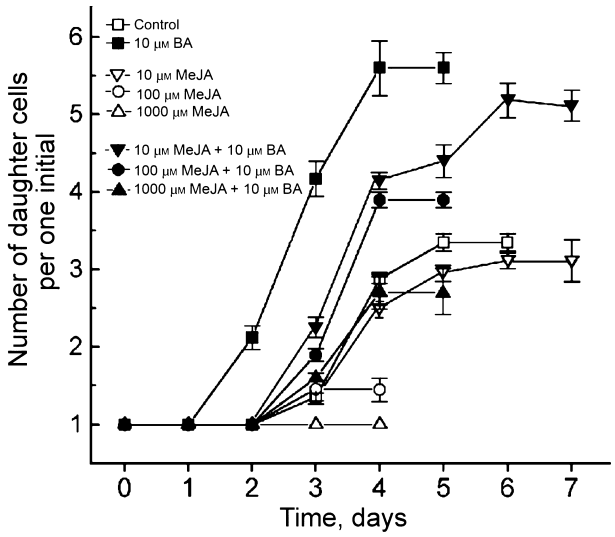

Fig. 3. Time course of palisade cell division as a function of time following excision. The various treatments are indicated in the legend. Data are mean ± SE of three replicate experiments.

mass as the large subunit of ribulose-1,5-bisphosphate carboxylase (rubisco, EC 4.1.1.39), as well as of polypeptides below 43 kDa, including bands at the same relative mass as reserve globulins (20–25 kDa) (Hara & Matsubara 1980). In contrast, benzyladenine eliminated almost completely the appearance of two polypeptides (33.5 and 32.6 kDa) that were present in the other treatments and stimulated the accumulation of the putative rubisco large subunit and a band at 13.4 kDa, both of which were decreased by jasmonate. Despite these differences, cytokinin, like jasmonate, decreased the bands in the area of the reserve globulins. When applied together at equimolar concentration (10 μM), the protein profile was similar to that of cotyledons treated with cytokinin alone; however, when jasmonate was given at higher concentration, the profile resembled jasmonate-only treatment.

DISCUSSION

Here, we report how the growing zucchini cotyledon responds to methyl jasmonate in comparison to cytokinin; additionally, we characterised the interaction between the hormones. We used cotyledons isolated from dry seeds and incubated in darkness. We found that jasmonate and cytokinin exert a contrasting suite of responses, usually, but not always, antagonistic.

The zucchini cotyledon appears to tolerate higher concentrations of jasmonate compared to other plant material. Most of the responses to methyl jasmonate that were

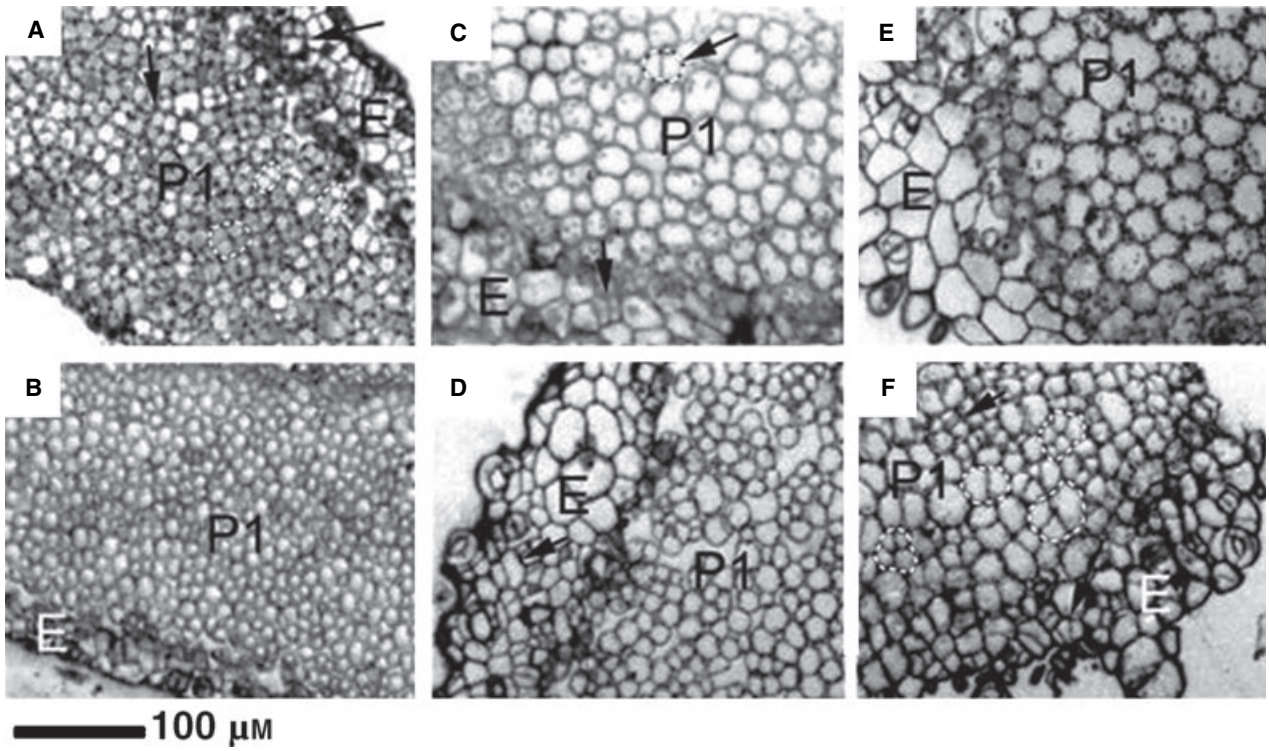

Fig. 4. Micrographs of paradermal sections (made at an acute angle of about 10̊ toward the cotyledon blade surface) showing the effects of treatments on palisade cell division and differentiation on day 6 of development. A: Control (water). B: 10 μM BA. C: 100 μM MeJA. D: 100 μM MeJA and 10 μM BA. E: 1 mM MeJA. F: 1 mM MeJA and 10 μM BA. P_1 = first palisade layer; E = upper epidermis; arrow = cluster of daughter cells. Dashed white lines highlight representative cell clusters.

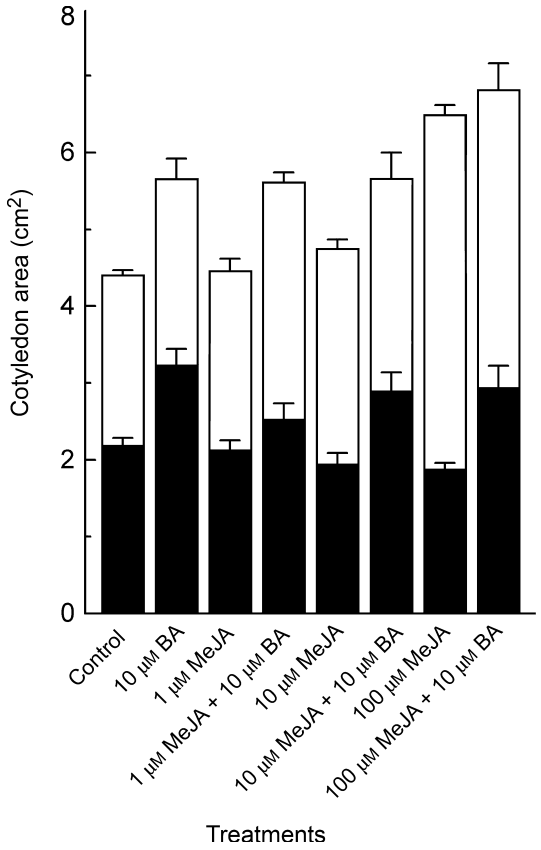

Fig. 5. Cotyledon area as a function of treatment. Areas were measured when cell number per cluster became constant (black bars) and at the end of the experiment (white bars). Data are mean ± SE of three replicate experiments.

assayed required at least 10 μM to elicit, and were more extreme on 1 mM. At the latter concentration, although the cotyledons sometimes had a few small spots, mostly they appeared normal and were able to expand vigorously. In contrast, 50 μM methyl jasmonate induced necrotic lesions in grape leaves (*Vitis vinifera*; Repka *et al.* 2004). This suggests that the cotyledon is adapted to high concentrations of jasmonate during etiolated growth.

Table 1. Cotyledon fresh and dry weights after 6 days of cultivation.

treatment	fresh weight, mg	dry weight, mg
dry seed cotyledon	60 ± 2***	40 ± 0.9
control	207 ± 12	39 ± 1.6
10 μM BA	311 ± 15***	40 ± 1.7
1 mM MeJA	250 ± 8***	33 ± 1.4**
1 mM MeJA + 10 μM BA	390 ± 8***	36 ± 1.5*

Data are mean ± SE. Asterisks indicate significance: ***P < 0.001; **P < 0.01; *P < 0.05.

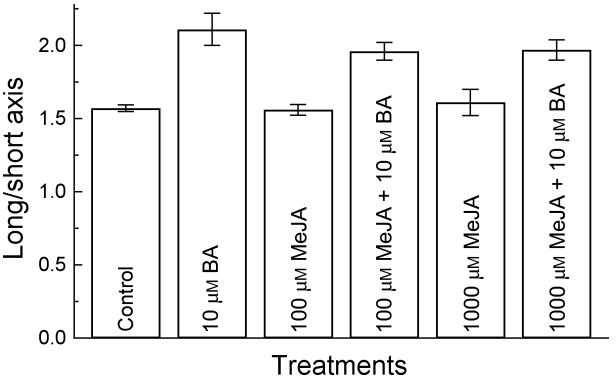

Fig. 7. Cotyledon aspect ratio on day 6 of cultivation as a function of the various treatments. Bars represent means ± SE of 25 replicates.

Methyl jasmonate stimulated expansion in a concentration-dependent manner, and concomitantly inhibited cell division. The role of cell division as a driving force in morphogenesis has long been debated (reviewed for leaf morphogenesis in Fleming 2002; Tsukaya 2006). There are many examples where mature organ size appears to depend on the number or rate of cell divisions; however, there are other examples where organ size appears independent of cell division. Examples of the latter presumably require some kind of compensatory mechanism that

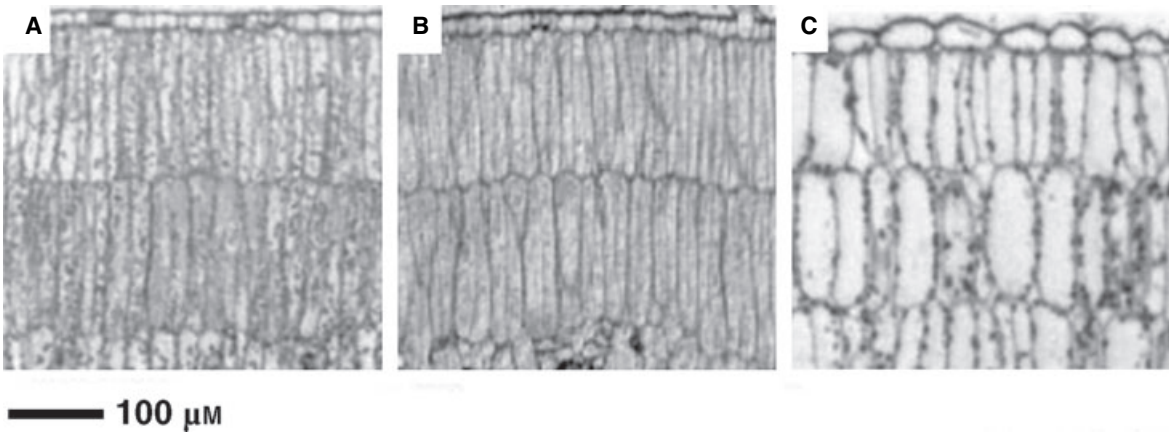

Fig. 6. Micrographs of palisade layers viewed in transverse sections. Day 6 of cultivation. A: Control (water). B: 10 μM BA. C: 100 μM MeJA.

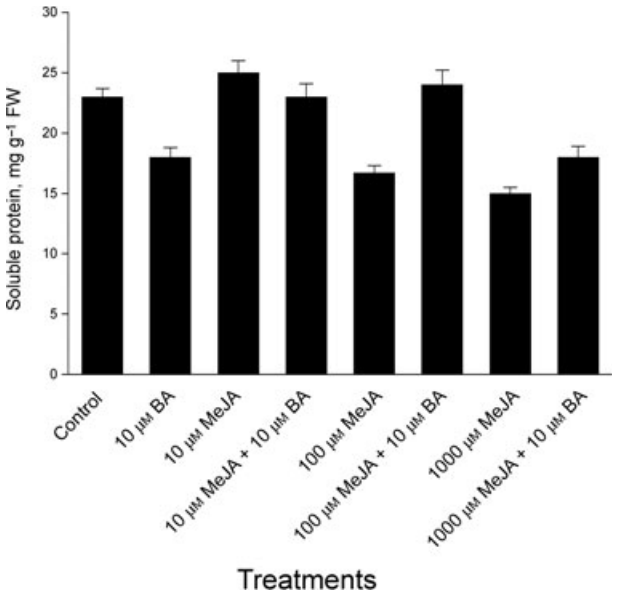

Fig. 8. Soluble protein content in palisade cells. Cotyledons were treated as indicated and cultured for 3 days in the dark. Palisade tissue was excised as described in Materials and methods. Protein content is expressed per g tissue fresh weight (FW). Data are means (±SE) of three replicates from a typical experiment. Two independent experiments gave comparable results.

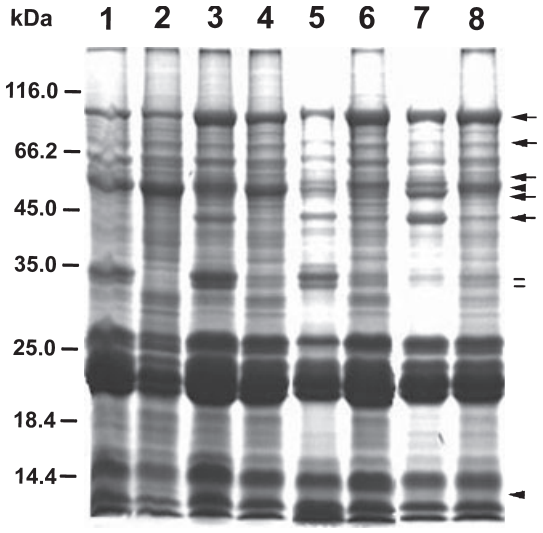

Fig. 9. SDS-PAGE analysis of polypeptide profiles of palisade tissue from cotyledons treated for 3 days in the dark. Treatments were as follows: lane 1, water (control); lane 2, 10 μM BA; lane 3, 10 μM MeJA; lane 4, 10 μM MeJA and 10 μM BA; lane 5, 100 μM MeJA; lane 6, 100 μM MeJA and 10 μM BA; lane 7, 1000 μM MeJA; lane 8, 1000 μM MeJA and 10 μM BA. Arrows point to MeJA-induced polypeptides at 97.4, 69, 60, 53 and 43 kDa; arrowheads indicate polypeptides increasing with BA; and solid lines point to polypeptides decreasing with BA. The molecular mass of markers is indicated to the left in kDa.

adjusts cell expansion to compensate for the production of too many or too few cells. The zucchini cotyledon response to jasmonate is a clear example of organ size being regulated independently of division: cells in the palisade layer underwent many (cytokinin-treated) or very few divisions (methyl jasmonate-treated) and there was only a slight difference in the final size of the cotyledon.

In terms of effects on growth, jasmonates are usually inhibitory (Wasternack & Hause 2002) and the pronounced stimulation of cotyledon expansion seen here is unusual. Another example where jasmonates stimulate expansion is in the formation of potato tubers (Cenzano *et al.* 2003). Tuberisation involves a loss of expansion anisotropy as the subapical stolon tissues swell, and in this way could be related to the more or less isotropic pattern of expansion seen here for the zucchini cotyledon. Interestingly, in tobacco cells, exogenous jasmonates cause reorientation or disruption of cortical microtubules (Abe *et al.* 1990), and in *A. thaliana* seedlings, elevated levels of endogenous or exogenous jasmonates decrease the amount of cellulose contained in root cell walls (Ellis *et al.* 2002). Both cortical microtubule disorganisation and decreased cellulose content are associated with decreased expansion anisotropy (Baskin 2005). During the jasmonate-stimulated expansion of cotyledons, it would be informative to examine cortical microtubules and cellulose microfibrils.

Our finding that methyl jasmonate inhibits cotyledon cell division is consistent with findings on the root meristem (Swiatek *et al.* 2003) and on tobacco BY-2 cells (Swiatek *et al.* 2002). The inhibition of cell division by jasmonate in tobacco BY-2 cells was not counteracted by exogenous zeatin; here, in contrast, benzyladenine restored division activity that was inhibited by methyl jasmonate, at least to some extent. Antagonism between cytokinin and jasmonate in regulation of cell division was also reported for callus (Ueda & Kato 1982).

In contrast to the antagonistic interaction seen for cell division, cytokinin and jasmonate both promoted cotyledon expansion. This differs from a report on radish cotyledons, where jasmonates antagonised the effect of cytokinin on expansion, as assayed as fresh weight gain (Ueda & Kato 1982). The reason for the difference between radish and zucchini is not clear, but Ueda & Kato (1982) used light-grown material and treated the cotyledons several days after germination, whereas we used etiolated material and treated cotyledons immediately after excision from dry seeds. Sensitivity of zucchini cotyledons to jasmonic acid may change with time from germination insofar as fresh weight gain in cotyledons excised 4 day after germination was insensitive to jasmonate (Ananieva & Ananiev 1999) whereas cotyledons excised from dry seeds responded (Table 1).

Methyl jasmonate, although stimulating cotyledon expansion, caused a somewhat isotropic mode of expansion and appeared to accelerate senescence, as indicated by the disappearance of cellular inclusions (presumably

storage bodies), the presence of of damaged tissue, and the substantial decrease in dry weight and soluble protein content. Although none of these are unambiguous indicators of senescence, jasmonates have been found to enhance senenscence in a variety of systems (Mukherjee *et al.* 2002; Wasternack & Hause 2002; Hung & Kao 2004). In contrast, cytokinin tended to accentuate the early phases of development, as evidenced here by the earlier onset of division and the earlier attainment of final cotyledon area. Cytokinin caused a somewhat anisotropic mode of expansion and made the cotyledon blades undulate, which may reflect excess cell divisions insofar as a similar phenotype is observed in *Arabidopsis thaliana* leaves following the over-expression of a cyclin D (Riou-Khamlichi *et al.* 1999). Undulations were absent from jasmonate-treated blades.

We demonstrate here that excised zucchini cotyledons responded to jasmonate treatment by the induction of three proteins, and the accumulation of two other proteins (Fig. 9). Similar bands were reported previously for zucchini cotyledons excised 4 day after germination, incubated on water alone for 1 day, and then treated for 1 to 4 day with jasmonate (Ananieva & Ananiev 1999). However, we also found that jasmonate decreased the abundance of a band migrating at the same relative molecular mass as the large subunit of rubisco, a decrease that is correlated with senescence. Additionally, we found that benzyladenine was unable to completely neutralise the effect of jasmonate on the induction of specific proteins. In barley and rice leaves, along with promoting senescence, jasmonates reduced synthesis of both large and small subunits of rubisco (Weidhase *et al.* 1987; Reinbothe *et al.* 1993; Rakwal & Komatsu 2001), and in barley leaves, cytokinin added with jasmonate similarly prevented senescence symptoms but not the accumulation of jasmonate-specific polypeptides (Weidhase *et al.* 1987).

Cytokinins are well known stimulators of expansion in cotyledons (Thomas *et al.* 1981); we suggest that they act on expansion by a mechanism that is at least partially distinct from that of jasmonate. Jasmonate led to faster consumption of stored reserves; in addition, effects on the aspect ratio of the cotyledon and on the thickness of the palisade layer differed between the two growth regulators. In view of the importance of cotyledon function for the establishment of the seedling, it is reasonable that growth processes are subject to a network of finely tuned interactions. Continued elucidation of these interactions is a challenge for the future.

ACKNOWLEDGEMENTS

The Bulgarian National Foundation for Scientific Research (Contract B-1406) and the project PISA supported this work. P. P. performed some of the experiments under the direction of E. S.-B. The manuscript was written by E. S.-B and T.I.B.

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